UNIVERSITI PUTRA MALAYSIA

MOLECULAR STUDIES OF VIBRIO CHOLERAE STRAINS ISOLATED FROM LOCAL OUTBREAK

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By

WAN SOMARNY BT. WAN MD. ZAIN

Thesis Submitted to the School of Graduate Studies, Universiti Putra Malaysia in Fulfilment of the Requirement for the Degree of Master of Science

July 2003

To Daddy , Mummy, sisters, brothers, in laws, and beloved husband,
Words cannot express how much I love you all and how blessed I am to have you in my life. Thank you for seeing me through with your loves, strengths and prayers.

To my friends:
Norazila Kassim Shaari, Azizah Mohd. Taib, Azlina Mohd. Daniel, Nur Asma Ariffin, Samsiah Otoi, Ruzainah Bt. Ali, Nurmawati Syahroni, Shariza Nordin Sharizah Alimat and Norin Zamiah Kassim Shaari

We are not lovers
because of the love
we make
but the love
we have

We are not friends
because of the laughs
we spend
but the tears
we save

I don't want to be near you
for the thoughts we share
but the words we never have
to speak

I will never miss you
because of what we do
but what we are
together

"Thanks for Everything"

Abstract of thesis presented to the Senate of Universiti Putra Malaysia in fulfilment of the requirements for the degree of Master of Science

MOLECULAR STUDIES OF *VBRIO CHOLERAE* STRAINS ISOLATED FROM LOCAL OUTBREAK

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July 2003

Chairman : Associate Professor Mariana Nor Bt. Shamsudin, Ph.D.

Faculty : Medicine and Health Sciences

Cholera caused by toxigenic *Vibrio cholerae* is a major public health problem in developing countries. Epidemiology surveillance of cholera and comparative analysis of strains collected during outbreaks has demonstrated clonal diversity among epidemic strains and a continual emergence of new clones of toxigenic *V. cholerae*. In the present study, the Random Amplified polymorphic DNA (RAPD) technique was performed to study the clonal diversity on twenty isolates of *V. cholerae* and five isolates of *V. harveyi* (as a comparison). Eighteen of *V. cholerae* isolates belong to the Ogawa serotypes, while two isolates were Inaba and 0139 Bengal serotypes, respectively. Fourteen out of twenty random primers yielded clear and reproducible bands. From the RAPD banding profiles, the polymorphism rates of bands were much higher between *V. cholerae* and *V. harveyi* than those found among *V. cholerae* isolates. According to the dendrogram generated from the RAPDistance software program,

V. cholerae and *V. harveyi* isolates were distinctly separated into their own groups. The Nei and Li's genetic distance obtained in this study ranged from 0.024691-0.644860 among *V. cholerae* isolates and 0.25368 - 0.633028 among *V. harveyi* isolates. The percentage of similarity among all *V. cholerae* isolates ranged from 32.5% to 99.4% and among *V. harveyi* isolates ranged from 32.5% to 73.0%. Analysis of RAPD bands using GEL COMPAR software at 50% similarity level could distinguish these isolates as well as Inaba and Bengal serotypes.

In this study, the Accessory cholerae enterotoxin (*ace*) gene, the third toxin of the *V. cholerae* virulence cassette was successfully amplified and isolated from 15 out of 20 local outbreak isolates of *V. cholerae*. However, this gene could not be amplified from the DNA in any of the five *V. harveyi* isolates. The amplification of *ace* gene produced a single band of 314 bp. In addition, the lipopolysaccharide (LPS) biosynthesis gene, *rfaz* was also successfully amplified in all *V. harveyi* isolates and 7 of the *V. cholerae* isolates whereby a single fragment of 1.2 kb was amplified. The specificity of the amplified products, *ace* and *rfaz* genes were then confirmed by the Southern hybridization technique using Ace and ZB3 biotinylated probes. The Southern hybridization results showed that all the PCR products amplified by *Ace*I/*Ace*II and *ZPf*/*ZPr* primers are specific to Ace and ZB3 probes, respectively.

In order to allow the production of large quantities of the DNA fragment for physical and biology analysis, the *ace* and *rfaZ* genes were cloned into 2.1 TOPO TA vector and were transformed into TOP10, *E. coli* host strain. From the sequencing analysis using BLASTN package, several mutations were found in the sequence, resulting frameshift mutation. Due to the mutation in the *ace* gene sequence in 2.1 TOPO TA vector, a new expression vector pBAD/Thio TOPO was chosen to clone the *ace* gene. Since the gene was found to be toxic, the positive construct, pBAD10/*ace*2 (*E. coli* TOP10 as a host strain) was retransformed into another *E. coli* host strain, LMG194 which produced a new construct, pBADLMG/*ace*2. Both of the constructs, pBAD10/*ace*2 and pBADLMG/*ace*2 were successfully transcribed by the detection of the *ace* gene in mRNA samples by Reverse transcriptase-PCR (RT-PCR). However, the pBAD10/*ace*2 construct failed to express the *Ace* protein. On the other hand, the pBADLMG/*ace*2 construct successfully expressed the soluble *Ace* protein even though there was an addition of two bases, T and G after the first ATG in the *ace* gene sequence. Analysis using the Biology Workbench 3.2 showed that the start codon is GTG, encoding the amino acid valine instead of methionine. The *Ace* fusion protein with the expected size of approximately 34 kDa was expressed after 72 hours induction with 0.02% arabinose.

Abstrak tesis yang dikemukakan kepada Senat Universiti Putra Malaysia sebagai memenuhi keperluan untuk ijazah Master Sains

KAJIAN MOLEKULAR KE ATAS *VIBRIO CHOLERAE* STRAIN PENCILAN DARI WABAK TEMPATAN

Oleh

WAN SOMARNY BT WAN MD. ZAIN

July 2003

Pengerusi : Profesor Madya Mariana Nor Shamsudin, Ph.D.

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Penyakit kolera yang disebabkan oleh *Vibrio cholerae* yang toksigenik merupakan masalah kesihatan yang utama di negara membangun. Kajian epidemiologi kolera dan analisa perbandingan strain yang dikumpul sepanjang wabak menunjukkan kepelbagaian klonal terhadap strain epidemic dan kewujudan klon baru *V. cholerae* yang patogenik secara berterusan. Dalam kajian ini, teknik DNA polimorfik menggunakan primer rawak (RAPD) diaplikasikan untuk mengkaji kepelbagaian klonal bagi 20 isolat *V. cholerae* dan 5 isolat *V. harveyi* (sebagai perbandingan). Lapan belas isolat *V. cholerae* merupakan serotip Ogawa, sementara dua isolat yang masing-masing merupakan serotip Inaba dan 0139 Bengal. Empat belas daripada duapuluh priner rawak menghasilkan jalur yang terang dan kebolehulangan. Dari profil jalur RAPD kadar polimorfisma adalah lebih tinggi di antara *V. cholerae* dan *V. harveyi* berbanding yang ditemui di antara isolate *V. cholerae*. Merujuk kepada dendrogram yang dihasilkan dari

program RAPDistance, isolat *V. cholerae* dan *V. harveyi* dibahagikan kepada kumpulan mereka tersendiri. Jarak genetik Nei dan Li yang diperolehi dari kajian ini, berjulat di antara 0.024691 – 0.644869 iaitu di antara isolat *V. cholerae* dan 0.025368 – 0.633028 bagi isolate *V. harveyi*. Peratus kesamaan di antara isolat *V. cholerae* adalah berjulat 32.5% hingga 99.4% manakala antara isolat *V. harveyi* adalah berjulat dari 32.5% hingga 73.0%. Analisis jalur RAPD dengan menggunakan software GEL COMPAR menunjukkan nilai kesamaan pada tahap 50% berupaya untuk membezakan isolat – isolat ini dan begitu juga serotip Inaba dan Bengal.

Dalam kajian ini, gen *ace* iaitu yang merupakan toksin ketiga bagi virulen kaset *V. cholerae* telah berjaya diamplifikasikan dan diisolat iaitu sebanyak 15 dari 20 keseluruhan isolat serangan tempatan bagi *V. cholerae*. Namun begitu, gen ini tidak diamplifikasikan dari DNA kelima – lima isolat *V. harveyi*. Amplifikasi gen *ace* menghasilkan satu jalur tunggal pada 314 bp. Di samping itu, gen biosintesis lipopolisakarida (LPS), *rfaZ* juga berjaya diamplifikasikan dalam kesemua isolat *V. harveyi* dan 7 isolat *V. cholerae* di mana satu jalur tunggal pada 1.2 kb telah diamplifikasikan. Spesifikasi terhadap produk amplifikasi, gen *ace* dan *rfaZ* kemudiannya dipastikan dengan teknik 'Southern hybridization' menggunakan probe Ace dan ZB3 yang dilabelkan dengan biotin. Keputusan 'Southern hybridization' menunjukkan kesemua produk PCR yang diamplifikasikan oleh primer *AceI*/*Ace*II dan *ZPf*/*ZPr* adalah spesifik terhadap probe Ace dan ZB3.

Untuk membenarkan penghasilan jalur DNA dalam kuantiti yang banyak untuk analisis fizikal dan biologi, gen *ace* dan *rfaZ* telah diklonkan dalam 2.1 TOPO TA vektor dan ditransformasikan ke dalam TOP10, strain perumah *E. coli*. Daripada analisi penjujukan dengan menggunakan pakej BLASTN, terdapat beberapa mutasi telah ditemui dalam jujukan yang menghasilkan mutasi 'frameshift'. Berdasarkan kepada mutasi dalam jujukan gen *ace* di dalam vector 2.1 TOPO TA, satu vektor ekspresi baru iaitu pBAD/Thio TOPO telah dipilih untuk pengklonan gen *ace*. Memandangkan gen tersebut adalah toksik, pembentukan positif pBAD10/*ace*2 telah ditransformasi sekali lagi ke dalam strain perumah *E. coli* yang lain iaitu LMG194 di mana pembentukan yang baru dihasilkan iaitu pBADLMG/*ace*2. Kedua-dua penghasilan ini pBAD10/*ace*2 dan pBADLMG/*ace*2 telah berjaya ditranskripsikan dengan pengenalpastian gen *ace* dalam sampel mRNA dengan menggunakan kaedah transkripsi berbalik – PCR (RT-PCR). Walaubagaimanapun, pembentukan pBAD10/*ace*2 telah gagal untuk mengekpres protein *Ace*. Sebaliknya, pembentukan pBADLMG/*ace*2 telah berjaya mengekpres protein *Ace* larut walaupun dengan penambahan dua bes, T dan G selepas ATG yang pertama dalam jujukan gen *ace*. Analisis dengan Biology Workbench 3.2 menunjukkan kodon permulaan ialah GTG yang mengkodkan asid amino valine selain dari methionine. Protein *Ace* yang bersaiz kira-kira 34 kDa diekspresikan setelah 72 jam selepas menginduksikannya dengan 0.02% arabinose.

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I certify that an Examination Committee met on 24th July 2003 to conduct the final examination of Wan Somarny bt. Wan Md. Zain on her Master of Science thesis entitled "Molecular Studies of *Vibrio cholerae* Strains Isolated from Local Outbreak" in accordance with Universiti Pertanian Malaysia (Higher Degree) Act 1980 and Universiti Pertanian Malaysia (Higher Degree) Regulations 1981. The Committee recommends that candidate be awarded relevant degree. Members of the Examination Committee are as follows:

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This thesis submitted to the Senate of Universiti Putra Malaysia has been accepted as fulfilment of the requirements for the degree of Master of Science. The members of the Supervisory Committee are follows:

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DECLARATION

I hereby declare that the thesis is based on my original work except for quotations and citations which have been duly acknowledged. I also declare that it has not been previously or concurrently submitted for any other degree at UPM or other institutions.

WAN SOMARNY BT. WAN MD. ZAIN

Date : 10/11/2003

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LIST OF ABBREVIATIONS

ace	accessory cholera enterotoxin
AP-PCR	arbitrary primed polymerase chain reaction
APS	ammonium persulphate
ASW	artificial sea water
ATCC	American type culture collection
bp	base pair
CA	cytophaga agar
cAMP	cyclic adenosine 51-monophosphate
cDNA	complementary DNA
CT	cholera toxin
CTX Φ	cholera-toxin phage
*ctx*A	cholera toxin A
DNA	deoxyribonucleic acid
EDTA	ethylenediamine tetraacetatic acid
GM1	monosialosyl ganglioside
GTP	guanosine 5'-triphosphate
H_2O_2	hidrogen peroxide
H_2O	water
HCl	hydrochloride acid
HRP	horseradish peroxidase
IF	initiation factors
IgA	immunoglobulin A
kb	kilo base pair
KCl	potassium cloride
kDa	kilo daltons
KDO	2-keto-3-deoxyoctonate
LB	luria bertani
LPS	lipopolysaccharide
$MgCl_2$	magnesium cloride

mRNA	messenger Ribonucleic Acid
NaCl	sodium chloride
NJTREE	neighbour joining Tree
OD	optical density
OMPs	outer membrane proteins
PCR	polymerase chain reaction
PEG	polyethylene glycol
PFGE	pulsed field gel electrophoresis
pmoles	picomoles
RAPD	Random Amplified Polymorphic DNA
RFLPs	Restriction Fragment Length Polymorphisms
RNA	ribonucleic acid
RS1	repetitive sequence
RT-PCR	reverse transcription-PCR
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate – polyacrylamide gel electrophoresis
TBE	tris–borate-EDTA
TCBS	thiosulphate citrate bile salt sucrose
UV	ultraviolet
V	volt
WHO	World Health Organization
zot	zonula occludens toxin
µg	microgram
µl	microliter

CHAPTER ONE

INTRODUCTION

Vibrio cholerae is a highly motile gram-negative bacterium and is the causative agent of epidemic cholera. Cholera, a highly epidemic diarrheal disease, is caused by toxin – producing strains of *Vibrio cholerae* 01. *V. cholerae* of serogroup 01 produces a potent heat-labile enterotoxin, termed cholera toxin (CT). Cholera is caused by the action of cholera toxin, CT which through its stimulation of intestinal adenylate cyclase activity gives rise to the characteristic excessive electrolyte secretion and fluid loss from the small intestine (Osek *et al*., 1992). In its severe form, the disease can lead to fatal diarrhea dehydration and typically occurs in explosive epidemic whereby the small quantities of purified cholera toxin at 5 µg, can cause copius purging when fed to volunteers (Levine *et al*., 1983). In individuals infected with *V. cholerae*, cholera toxin is responsible for the drastic intestinal electrolyte secretion and fluid loss leading to the clinical state of cholera (Holmgren, 1981 ;Finkelstein, 1984). *V. cholerae* 01 are classified into two biotypes, classical and *El Tor*, and into two major serotypes, *Inaba* and *Ogawa* (Ghosh *et al*, 1996) while the Hikojima serotype has been rarely reported (Faruque *et al*., 1998). *V. cholerae* 01 is defined by agglutination in 0 group 1 in which 0 antigens (somatic antigens) is the target for the specific antiserum directed against the lipopolysaccharide component of the cell wall.

In 1992, cholera was caused by a new serotype 0139 synonym Bengal (Shimada *et al*., 1993). This serotype is the latest serogroup of *V. cholerae* that emerged in epidemic proportions in India and Bangladesh and is also known as a first non-01 group. *V. cholerae* non–01 serotypes are widely distributed in the aquatic environment and are free-living in nature. This serovar is identified by the absence of agglutination in 0 group 1 specific antiserum (Albert *et al*., 1993) but agglutinated in 0 group 139 specific antiserum and also by the presence of a capsule (Jonson *et al*., 199 *V. cholerae* non-01, were not known to be associated with such a large outbreak of diarrhea before the present of epidemic (Faruque *et al*., 1994). Moreover, this serotype was known to produce cholera toxin at a very low frequency (Janda *et al*., 1988). It has often been identified as the causative agent of sporadic cases (Karaolis *et al*., 1998 ;Russell *et al*., 1992) and localized outbreaks (Craig *et al*., 1981). However, in 1992 a 0139 serogroup strain emerged and caused epidemic disease throughout India and Bangladesh which replaced 01 strains of *V. cholerae* as the predominant cause of cholera on the Indian subcontinent (Albert *et al*., 1993 and Bhattacharya *et al*., 1993).

Strains of *V. cholerae* 01 impaired in their ability to colonize the gut are able to produce their symptoms of the disease and elaborate a powerful exotoxin, CT. Cholera toxin (84 kDa) is a fairly complicated molecule which is composed of one A subunit of 27 kDa and five B subunits, each of 11 kDa (Ghosh *et al*., 1996). Although CT is responsible for severe dehydrating diarrhea associated with *V. cholerae*, the search for the additional enterotoxin produced by